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NEURAL RECONNECTION IN THE TRANSECTED SPINAL CORD OF THE FRESH-WATER TURTLE *Trachemys dorbignyi*

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Abstract

This paper provides the first evidence that fresh water turtles are able to reconnect their completely transected spinal cord leading to some degree of recovery of the motor functions lost after injury. Videographic analysis showed that some turtles (5 out of 11) surviving more than 20 days after injury were able to initiate stepping locomotion. However the stepping movements were slower than those of normal animals and swimming patterns were not restored. Even though just 45% of the injured turtles recovered their stepping patterns, all showed axonal sprouting beyond the lesion site. Immunocytochemical and electron microscope images revealed the occurrence of regrowing axons crossing the severed region. A major contingent of the axons reconnecting the cord originated from sensory neurons lying in dorsal ganglia adjacent to the lesion site. The axons bridging the damaged region traveled on a cellular scaffold consisting of BLBP and GFAP positive cells and processes. Serotonergic varicose nerve fibers and endings were found at early stages of the healing process at the epicenter of the lesion. Interestingly, the glial scar commonly found in the damaged central nervous system of mammals was absent. In contrast GFAP and BLBP positive processes were found running parallel to the main axis of the cord accompanying the crossing axons.

Keywords

spinal cord; spinal cord injury; nervous regeneration; axon regrowth; brain lipid binding protein; serotonin

INTRODUCTION

Spinal transection in humans results in an irreversible loss of motor and sensory functions. This unfavorable condition is common to all mammals which are unable to reconnect neuronal pathways after severe spinal cord injury. However, exceptions occur during embryonic life since in marsupial embryos the transected cord heals as development proceeds, leading to the restoration of functions (Saunders et al. 1998). In a number of other vertebrates like cyclostomes (Rovainen, 1976; Wood and Cohen, 1979, Armstrong et al. 2003, reviewed by Shifman et al. 2007), some teleosts (Dervan and Roberts 2003; Takeda et al. 2007) and tailed amphibians (Piatt, 1955, Stensaas, 1983; Davis et al. 1990; Chevallier et al. 2004) the spinal cord seems to have self-repairing mechanisms that lead to total or partial recovery of sensory-motor functions. According to Stensaas (1983) “urodeles thus constitute

the most advanced phylogenetic group in which functional regeneration occurs following lesions that interrupt ascending and descending pathways of the spinal cord". Nevertheless, it is accepted that some reptiles like lizards, are able to regenerate amputated legs and tails (Guibé, 1970) including the terminal portions of the spinal cords (Egar et al. 1970). Despite recognition of the regeneration potentialities of reptiles there is a surprising lack of information about the responses to mid-trunk damage of the spinal cord. The occurrence of endogenous regenerative mechanisms in a taxon closer to mammals launches new opportunities for identification of key mechanisms subserving restoration of the insulted CNS. This includes the potential design of novel strategies for spinal cord repair.

The spinal cord of the turtle has been a useful model to study the generation of a variety of motor patterns at the systems level (Stein, 2008). In addition, the outstanding resistance of freshwater turtles to hypoxia (Lutz et al. 1985) enabled the use of in vitro preparations of mature spinal networks to provide insights on cellular and synaptic mechanisms (Hounsgaard and Nicholson, 1990; Russo and Hounsgaard, 1996 a-b) later shown to be preserved in mammals (Morisset and Nagy, 2000; Hultborn et al. 2004). Despite anatomical and functional similarities, the spinal cord of turtles has some features that its mammalian counterpart seems to lack. For example, the turtle spinal cord retains the ability to generate new neurons after birth (Fernández et al., 2002). We have reported that this ability is due to the presence of cells located on the lateral aspects of the central canal (CC) that have the anatomical, molecular and functional properties of neurogenic precursors similar to those in the embryo and neurogenic niches of the adult mammalian brain (Russo et al., 2004; Trujillo-Cenóz et al., 2007; Russo et al., 2008). We hypothesize that the persistence of these precursors within functionally matured spinal circuits may endow turtles with efficient mechanisms for structural plasticity in response to injury.

In the present article we provide the first evidence that a free-living amniote -the fresh-water turtle *Trachemys dorbignyi*- reconnects the completely transected spinal cord and recovers some of the motor functions lost after injury. Our findings, based on videographic analysis, immunohistochemistry and electron microscopy, revealed that spinal cord damage is partly repaired by the formation of a scaffold of glial cells and processes that support the transit of regenerating axons. Collectively, the results presented here indicate that fresh-water turtles fulfill the basic requirements proposed by Becker and Becker (2007) to constitute a suitable vertebrate model system for studying spinal cord damage and repair. The introduction of this new model of spinal cord injury will contribute to identify phylogenetically old repairing mechanisms in reptiles, that function to a lesser extent in rodents (Houle and Yin, 2001; Iseda et al. 2004; Bareyre et al. 2004) and at a bare minimum in humans (Guest et al. 2005).

MATERIAL AND METHODS

Animals

Fresh-water turtles (*Trachemys dorbignyi*, carapace lengths ranging between 5-12 cm) were used (n= 25). These small turtles (2 months to 1 year old) behave like fully mature animals in terms of their sensory-motor behavior. They were maintained in temperate aquaria (24-26° C) under natural illumination and daily fed with small earthworms. All the studies were conducted under the guidance of our local Committee for Animal Care and Research (CHEA, UDELAR), which follows NIH guidelines for maintenance and use of laboratory animals.

Anesthetics and surgical procedures

Anesthesia was used for all surgical procedures and also before intraventricular perfusion with fixative solutions. For spinal cord transection we first induced animal sedation by

intraperitoneal injection of ketamine chlorhydrate (40 mg/kg, b.w.). Then, the four legs were secured to a rigid support by means of rubber bands and complete anesthesia achieved by inhalation of isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether, Forane®, Abbot Laboratories England). To prevent infection we rubbed the carapace with a cotton-pad previously immersed in an alcohol-iodine solution whereas surgical instruments were sterilized using chemical procedures. After the animal was unresponsive to nociceptive stimuli, we opened a window in the carapace following the limits of the third dorsal scute to expose the spinal cord. A complete transection of the spinal cord was performed with a thin-blade scalpel (Fig.1A). Following transection of the cord there was a consistent retraction and separation of the resulting stumps, and thus the chances of axonal sparing were minimal (Fig.1 B). After washing the injured site with sterilized saline we replaced the lifted scute and sealed the injured carapace with cyanocrylate adhesive.

Postoperative care

We identified the spinalized turtles with indelible marks sculptured on the carapace. Turtles do not require special postoperative care because, as described for other non-mammalian vertebrates (Mountcastle, 1980), spinal shock is extremely short. Since turtles have a primitive “allantoic bladder” opening into the cloaca (Parker and Harwell, 1949) there is not noticeable urine retention. Despite their reduced motor capabilities operated turtles were able to catch living worms a few days after surgery.

We studied a total of 25 turtles that were killed at the following time intervals after the lesion: 1) between 24-28 h: n=3; 2) between 5-7 days: n=6; 3) between 15-17 days: n=5; 4) between 20-40 days: n=5 (1 turtle recovered stepping locomotion); 5) between 60-120 days: n=6 (four turtles recovered stepping locomotion).

Videographic analysis

To analyze the motor behavior of normal and spinalized turtles we made video recordings at 30 frames/s using a digital camera. Motor behavior was studied in free-swimming and free-stepping conditions and also in stationary experiments in which the animal body was secured to a support that did not interfere with the movements of the legs. The later experiments were directed to assess the role played by proprioceptive stimuli during the motor recuperation process. The video recordings of stationary swimming movements were performed in turtles with their ventral body surfaces secured with cyanocrylate to a heavy support immersed in the aquarium. The movements of the legs in a stationary position were also recorded by suspending turtles in the air by gluing the dorsal surface of the carapace to a rigid bar. The resulting movies were analyzed frame by frame with a time resolution of 0,04 s with commercially available software (Windows Movie Maker®).

General Histological Procedures

The spinal cords of anesthetized turtles (5 mg/kg sodium methohexitone, i.p.; Brietal; Lilly) were fixed by perfusion through the heart. For immunocytochemistry (n=25) we used 10% paraformaldehyde dissolved in 0.1M phosphate buffer (PB, pH 7.4). Fixed material was sectioned with a vibrating microtome following planes oriented either perpendicularly or longitudinally respect to the major axis of the cord.

For transmission electron microscopy (TEM) (n=4: 28 h, 7, 15 and 54 days) some sections were post-fixed with glutaraldehyde (1% in PB) followed by a second fixation with OsO₄ (2% in PB). Sequential triple post-fixation (paraformaldehyde, glutaraldehyde, osmium) allowed the alternated exploration of spinal cord sections with both immunohistological techniques and TEM.

Dehydration was carried out in the alcohol series and the sections were finally embedded in epoxy resin. Semithin sections (1 μ m thick) were stained with borax methylene blue (BMB). Series of ultrathin sections were mounted on Formvarcoated slot grids (2 $\times$ 1 mm) and contrasted with uranyl acetate and lead citrate. We examined several series of sections (50-200 sections) at different magnifications with a Jeol 100CX electron microscope.

Immunocytochemistry

Antibody Characterization—Please see Table 1 for a list of all antibodies used.

The anti-neurofilament M (NF-M) antibody recognizes a band of 145 kDa evaluated by Western Blot on mouse brain lysates, and reacts with different species including “alligators and tortoises” (manufacturer’s datasheet). Harris et al. (1991) showed by immunoblotting that the NF-M antibody strongly recognizes NF-M but not NF-H in the brain of cow, pig and chicken. As demonstrated in several species (rabbit, rat, chicken, fish, turtle, and frog) by Schachner et al. (1978), anti-neurofilament protein antiserum only reacts with neurons. In agreement, NF-M in the turtle spinal cord reacts with cells that express neuronal markers (Russo et al., 2004). Our images closely resembled those shown by Harris et al. (1991) in neurons of the brainstem, cerebellum and brain of the rat.

According with the compatibility of the antibodies in double labeling experiments, we used anti-glial fibrillary acidic protein (GFAP) antibodies raised in mouse or rabbit. Both the monoclonal and polyclonal antibodies recognized a single band of ~50 kDa on Western blots performed in the human cortex (Toro et al., 2006) and rat retina (Chang et al., 2007), respectively. They were tested by us in mammalian and quelonian nervous systems (Trujillo-Cenóz et al., 2007), producing a staining pattern comparable to that described by Talos et al. (2006).

Anti-brain lipid binding protein (BLBP) antibody recognized a single band of ~15 kD on Western blots of mouse cerebellum and forebrain (manufacturer’s datasheet, see also Feng et al., 1994). It has been reported that the antibody stains a population of cells with the typical morphological features of radial glia (RG) (Feng et al. 1994; Kriegstein and Götz, 2003). In a previous paper (Russo et al. 2008), we demonstrated that the antibody reacts with a subpopulation of RG in the spinal cord of turtles.

Anti-5-HT antibody cross-reactivity was examined with 5 μ g, 10 μ g, and 25 μ g 5-hydroxytryptophan, 5-hydroxyindole-3-acetic acid, and dopamine. Negative results were obtained with antisera diluted 1/20,000 (Bn-SA/HRP labeling method; manufacturer’s technical information). No staining was observed when the antiserum was preincubated with 1.5 mM serotonin (Thompson et al., 1994). We also tested the antibody in well-known serotonergic neuronal systems such as the raphe nuclei of turtles and the nerve cord of isopods. The patterns of neuronal and axonal staining were identical to those described for turtles (Kiehn et al., 1992) and isopods (Thompson et al., 1994).

Anti-zonula occludens-1 (ZO-1) antibody detects ZO-1 α + and ZO-1 α - isoforms on Western blots analysis of T84 cell lysates (manufacturer’s datasheet). Western blots obtained from goldfish brain show that the ZO-1 antibody recognizes a single band at the predicted molecular weight for this protein (Flores et al., 2008a). The specificity of the ZO-1 antibody was confirmed in the spinal cord of turtles because it stained the junctional complexes occurring in the apical segments of the cells that line the CC. The antibody was kindly donated by Dr.A. Pereda (Dominick P. Purpura Department of Neuroscience, Albert Einstein College of Medicine).

In all cases non-specific reactive sites were blocked with 0.5% bovine serum albumin (BSA) in PB (1 h) followed by overnight incubation with the primary antibody (in PB and 0.3% Triton X-100). After blocking and washing, the tissues were incubated with goat anti-rabbit or goat anti-mouse secondary antibodies conjugated either with horseradish peroxidase (HRP, 1/200, 1h in the dark; Sigma grade VI) or fluorophores (Alexa 488 or 633; 1/1000, 2 h; Invitrogen, Eugene OR). HRP was visualized using -3',3' diaminobenzidine or peroxidase substrate kit (Vector SG substrate kit, Cat. SK 4700. Vector Laboratories). Control experiments replacing antibodies by pre-immune serum were routinely performed. To counter stain sections revealing nuclei characteristics, we employed the nucleic acid stain Syto 64 (Invitrogen Eugene OR, Cat, N S-11346, lot, 1881-3).

Axonal tracers

To identify the origin of the regenerating axons crossing the lesion, we used two technical procedures: 1) application of 1,1',di-octadecyl 1-3,3,3',3'-tetramethylcarbocyanine perchlorate (DiI) (Invitrogen) crystals on the first rostral sensory nerves respect to the lesion site (see Fig. 9 A, inset). We also applied DiI crystals on the cut surfaces of the cord 3-4 mm caudal to the lesion (see schematic drawing in Fig. 12) and 2) the iontophoretic injections of biocytin in the bridge joining the rostral and caudal stumps, followed by visualization of the labeled cells with streptavidine conjugated with horseradish peroxidase.

The carbocyanine dye was employed in aldehyde-fixed tissues (Godement et al.1987, Köbbert et al. 2000) that were maintained immersed in the fixative solution for 15 days at 37°C and finally sectioned with a vibrating microtome. The iontophoretic injections of biocytin were made in living tissues and the spinal cord was fixed 4h after injection and processed for light microscopy inspection. Independently from the technical approach, fluorescent signals were detected using epi-fluorescence or confocal microscopy (Olympus VF300). The images were imported into Fluoview 5 (Olympus) to generate z-stacks as well as orthogonal views and then exported to PhotoShop or Coreldraw for cropping and creation of figure montages. When necessary, the brightness and/or contrast of the images were adjusted.

RESULTS

Functional recovery after spinal cord transection

A key element of an animal model for studying spinal cord repair is whether the regenerative mechanisms are able to support some degree of functional recovery. To explore the process leading to potential motor recovery after complete transection of the spinal cord, we analyzed the normal and pathological repertoire of reflexes evoked by mechanical stimuli of the hindlimbs and tail. We also did simple videographic analysis of the locomotion patterns both in normal and spinalized turtles at different time points after spinal injury. It should be mentioned that as described for other non mammalian vertebrates (Mountcastle 1980) spinal cord transection was followed by a very brief spinal shock.

We found that in normal animals, pinching of the hindlimbs or tail evoked responses that varied from withdrawal of legs, tail and head retraction, to coordinated movements directed to remove the source of stimulation (Fig. 2 A). After recovery from anesthesia (4-6 h after spinal injury), spinalized turtles responded to tail pinching with an abnormal reflex consisting of the rapid bilateral adduction of the hindlimbs (0.49 ± 0.05 s; $n=3$) followed by a slower abduction and extension of the limbs (Fig. 2 B). We called the whole motor sequence the “tail-pinching reflex” and considered it a sign of spinal damage. Similar reflex movements of the hindlimbs were triggered by mechanical stimulation of the caudal border

of the carapace. It is important to note that the reflex persisted in all injured animals even though they had recovered stepping movements.

As expected, recently spinalized turtles were unable to move their hindlimbs reducing their ability for locomotion to a bouncy motion mediated by traction of the forelegs (Fig. 3 A, see supplementary movie). The paralyzed hindlimbs were usually maintained retracted under the carapace. However, when the animals were floating motionless in the aquarium, the paralyzed legs could be seen in an extended position. Under these circumstances, stimulation by tactile or visual stimuli, triggered evasive swimming maneuvers mediated by rapid, vigorous, strokes of the forelegs.

The first signs of motor recovery appeared in 45% of operated turtles beyond 20 days after surgery. They consisted of short-lasting stepping movements commonly involving one of the hindlimbs (Fig.3 B). At more advanced stages, turtles initiated stepping patterns exhibiting some coordination of forelimbs and hindlimbs (Fig.3 C, see supplementary movie). Our studies indicated that the time needed for a hindlimb to move from extension to flexion (a step) differed substantially between normal and spinalized turtles. Uninjured turtles were 6 to 10 times faster to step (0.17 ± 0.02 s; $n=4$) than the injured ones (1.01 ± 0.14 s; $n=5$). In addition, injured but stepping turtles were less resistant to fatigue than normal animals. The coordination between forelimbs and hindlimbs was evaluated by analyzing the sequence of stepping during spontaneous locomotion (10 step cycles). In normal animals ($n=4$), full extension of the left forelimb was followed by flexion of the right hindlimb with a mirror sequence on the right side. No missing steps were observed during the cycles analyzed and we thus considered this as 100% coordination. Although locomotion in regenerating animals was much slower, the coordination between forelimbs and hindlimbs was good. In one case the coordination was 100%, whereas in most turtles (3 out of 4) the hindlimbs skipped one or more step cycles (70, 80 and 90 % coordination). Moreover, the movements of the hindlimbs ceased when the animal was suspended in the air suggesting that proprioceptive feedback plays a key role in the genesis of recuperated stepping patterns. As described for salamanders (Chevallier et al. 2004), in no case coordinated swimming movements were redressed. The “swimming handicap” persisted even though the operated turtles had survived more than four months after complete transection of the spinal cord.

In spinalized lampreys, passive transmission of mechanical stimuli from rostral to caudal portions of the body supports certain amount of motor coordination (Wallen, 1982; Grillner and Wallen, 2007). Therefore, to demonstrate the occurrence of neural reconnection we performed immunohistochemical and TEM studies at various time points after the spinal cord injury.

The clot: initial bridging of the transected cord

Disruption of the meningeal sheaths together with damage to extrinsic and intrinsic blood vessels during spinal cord transection, destroys the hemato-encephalic barrier allowing a massive contact between nervous tissue and foreign cells and molecules. Invasion of hematogenous cells is now accepted as the source of secondary tissue damage (Jones et al. 2005). Nevertheless, the initial clot constitutes the first physical link between the recently separated spinal stumps (Figure 4 A). To investigate the structural organization of the clot and neighboring spinal regions, we studied the severed cord 28 h after injury. Our first technical approach was to stain with BMB semithin sections (1 μ m thick) of epoxy embedded tissues (Fig. 4 B). We found that in addition to conspicuous erythrocyte aggregates, the clot contains leukocytes with granules dispersed in the cytoplasm. Less frequently, there were cells devoid of granules with a faintly stained cytoplasm tentatively identified as macrophages. The morphology of the blood cells included in the clot was

similar to that described by Pitol et al. (2007) in the circulating blood of the turtle *Phrynosoma hilarii*. Interestingly, newly formed capillaries were found at the clot periphery indicating that reconstruction of the vascular bed is one of the earliest events involved in tissue repair (Fig 4 B). Next we examined ultrathin sections with TEM. The recorded images confirmed the occurrence of both erythrocytes and granulocytes (Fig 4 C), together with less numerous small-sized cells tentatively identified as lymphocytes (Fig.4 D). We also observed an extracellular matrix consisting of very thin fibrils (10 Å diameter) (inset in Fig. 4 D) that may contribute to the mechanical stabilization of the injured zone.

To investigate the early invasion of neural cells to the clot region we employed immunocytochemical procedures. The combined use of NF-M and GFAP antibodies indicated that 28 hours after injury, axons and glial processes were absent in the clot. However, preparations reacted with NF-M antibody showed in both the cephalic and caudal stumps numerous “dystrophic endbulbs” (Silver and Miller, 2004) ranging in size from 1 to 10 µm (Fig.4 E and F). Similar hypertrophied axon terminals were described by Ramón y Cajal (1913-14) in sectioned peripheral nerves and damaged nervous centers of mammals.

The formation of a cellular bridge

In a period ranging between 7-15 days after the spinal cord transection the clot was replaced by a bridge formed by other kinds of cells. The substitution process involves phagocytosis of erythrocytes that were seen partially fragmented and engulfed by large macrophages (Fig.5 A). There was also an increase in cell proliferation extending at both sides of the injury epicenter (not shown). However proper description and discussion of the cell proliferation process was beyond the goal of the present article and will be the subject of a separated report.

We hypothesized that replacement of the initial fragile clot by a more stable cellular bridge should imply the development of structures providing cohesiveness and compactness to the new cell populations. To test this, we examined the injured site with TEM. We detected typical desmosomes linking adjacent cells and processes (Fig.5 B and lower inset). To explore the occurrence of other membrane specializations that form part of the so-called junctional complexes we assayed the immunocytochemical identification of scaffold proteins of the tight junction family, also called ZO proteins. ZO-1 was strongly and specifically expressed at the level of the apical processes of the cells lining the CC (not shown). Consistent with the TEM findings we observed abundant ZO-1+ punctae (Fig.5 B, upper inset) as reported during premature cell compaction in mouse embryos (Winkel et al. 1990). Inspection of the cell processes at higher magnification showed a cytoplasmic matrix consisting of differently oriented microfilaments bundles (Fig.5 C). To investigate the different cell types populating the developing bridge, we explored the expression of GFAP and BLBP as markers of glial and RG precursor cells respectively, and neurofilament-M for axons and neurons.

As shown in figure 5 D exploration of early repairing stages disclosed the occurrence of two distinct cells classes: cells and processes expressing GFAP intermingled with cells and processes expressing BLBP. Remarkably, both kinds of processes ran parallel to the longitudinal axis of the spinal cord and did not form the entangled mass of reactive glia usually associated with failure of neuronal reconnection in the injured CNS of mammals (Ramón y Cajal, 1913-14). GFAP+ cell bodies were difficult to identify whereas the nuclei, cytoplasm and prolongations of cells expressing BLBP appeared robustly stained. Many of the BLBP+ cells had the typical morphology of migrating cells, with a single trailing-like process terminating by means of an enlarged foot (Fig. 5 E). It is worth to mention that cells with a similar morphology were routinely identified in our electronmicrographs (Fig. 5 F).

BLBP+ cells are selectively found around the CC of normal turtles and do not co-express GFAP (Russo et al., 2008). We tested whether changes in the molecular phenotype of BLBP + cells may occur at later stages and thus checked for co-localization of BLBP and GFAP. Indeed, we found that approximately 15 days after injury the bridge also contained cells co-expressing BLBP and GFAP (Fig. 6). GFAP predominated in the slender processes and peripheral cell regions while expression of BLBP was strongest in the nucleus and close perinuclear cytoplasm.

Bridge invasion by growing axons

Seven days after injury, unambiguous evidence of axon re-growth was scarce and mainly limited to sensory axons entering via the dorsal roots (Fig. 7 A). Interestingly, the growing axon-endings did not extend filopodia or lamellipodia as it occurs in typical growth cones of embryonic or cultured nervous tissue. Instead, axon elongation seemed to be mediated by smooth, bulb-like enlargements as reported by Shifman et al. (2007) in cyclostomes (for more details concerning the geometry of these endings see below).

Serotonergic signaling plays an important part in the generation of locomotor patterns and also during partial restoration of locomotion after spinal cord lesion (Nguyen et al. 2001, Orsal et al., 2002). For this reason, we explored the presence of 5-HT+ cells and fibers around the regenerating zone 7 days after the injury. We found that the epicenter of the injured site contained varicose axon terminals expressing 5-HT (Fig. 7 B). In agreement with the light microscope findings, the TEM images showed axon profiles containing -in addition to mitochondria and cumuli of clear vesicles- numerous dense-core vesicles of uneven size that suggested their monoaminergic nature (Peters et al. 1991) (Fig. 7 C). These 5-HT+ terminals appeared intermingled with other cells of the bridge without evidence of adherence points or clear postsynaptic specializations.

Approximately 15 days after injury, gross anatomical signs of spinal damage were difficult to detect either with the naked eye or when examined at a low power magnification (Fig. 8 A). We examined with TEM several series of ultrathin sections to investigate the structural relationships between the growing axons and the glial processes. At this stage of the healing process TEM images showed two kinds of processes: a) numerous round profiles exhibiting the fine structure features of thin unmyelinated axons (Fig. 8 B asterisks) and b) glial processes enveloping single axons or small axon bundles (Fig. 8 B, light-blue shadow). In addition, immunocytochemistry indicated the presence of axon growth cones expressing NF-M (Fig. 8 C, arrow). To reveal the shape of the growth cones, we made stacks of confocal optical sections that were processed to yield orthogonal x-y views. As already advanced, the growth cones were not flattened but cylindrical or conical in shape and practically devoid of filopodia and lamellipodia (Fig. 8 C inset). We also looked for axons expressing 5-HT at this more advanced stage of the repairing process. We found that instead of the scarce 5-HT+ varicose endings present at early time points after injury, the bridge region had now numerous serotonergic nerve axons branching profusely (Fig. 8 D).

Ramón y Cajal (1913-14) reported that in mammals the central branch of dorsal root ganglion neurons are able to invade the glial and meningeal scar. To assess whether sensory axons integrate the population of regenerating axons we employed a carbocyanine dye (DiI) as a suitable axon tracer to be used in aldehyde fixed nervous tissues (Godement et al. 1987). DiI crystals applied to the cut surfaces of sensory nerves lying close to the vertebral channel (see schematic drawing in Fig. 9 A) revealed that two weeks after injury few thin DiI labeled axons crossed the injured site (Fig. 9 A). Indeed, cross sections of the spinal cord passing through regions caudal to the lesion showed DiI-labeled processes in the periphery of the cord (Fig. 9 B1). Double labeling experiments incubating DiI-labeled material with NF-M antibody confirmed their axonal nature (Fig. 9 B2). Supporting the occurrence of

regenerative phenomena, both rostral and caudal stumps exhibited numerous axons with growth cones (Fig.9 C and inset).

Longitudinal sections performed 60 days after the lesion revealed numerous NF-M positive axon bundles bridging the gap between the rostral and caudal stumps. Most axons followed pathways parallel to the main axis of the cord (Fig.10 A). In agreement with the TEM findings made at earlier time points, double labeling experiments employing NF-M and GFAP antibodies indicated that the axons crossing the bridge were accompanied by GFAP+ processes (Fig.10 B and C). In addition, nuclear counter-staining with Syto revealed that these processes were part of the cellular scaffold supporting the reconnection of the cord (Fig.10 D and E).

In both the cephalic and caudal stumps we found neurons with a disorganized cytoskeleton (Fig 10 F) that were tentatively identified as damaged or dead neurons. However, we also found neurons with a normally stained cytoskeleton (Fig.10 G) that might have survived the secondary aggression generated by different classes of cytotoxins (Demopoulos et al. 1980, Jones et al. 2005). Moreover, preliminary patch-clamp recordings revealed the presence of healthy neurons able to fire repetitively close to the epicenter of the injured region (data not shown).

Transganglionic labeling experiments employing DiI, performed 120 days after the spinal cord lesion in a turtle that had recovered the movements of the hindlimbs (see videographic analysis section) showed the bridge populated by a major contingent of DiI labeled sensory axons stemming from labeled dorsal roots (Fig. 11 A-B). Moreover, sections caudal to the lesion site revealed DiI-labeled axon terminals contacting medium sized neurons with well developed NF-M+ cytoskeletons. The images were compatible with the occurrence of axo-somatic synaptic contacts (Fig. 11 C).

To determine whether local neurons whose axons were amputated by the lesion were able to regenerate, we applied DiI crystals on the exposed surface of the sectioned spinal cord caudal to the injury site (see scheme in Fig. 12). The spinal cord segments were maintained immersed in warm fixative (37 C°) for 15 days. The study of the corresponding sections revealed DiI-labeled propiospinal neurons lying at rostral levels respect to the lesion site (Fig. 12 A1-3). The most plausible interpretation is that the carbocyanine-dye molecules diffused along the respective axon membranes crossing the lesion site to reach and stain the perikarya. In addition, the iontophoretic injection of biocytin in the bridge (n=2, 15 days post injury) was also able to detect groups of retrograde labeled neurons in the cephalic stump, close to the lesion site (Fig. 12 B-C). In this case retrograde transport in living conditions was very rapid (less than 4 h). Since our goal was limited to demonstrate neural reconnection, we did not explore DiI or biocytin labeling during longer incubation times and the potential labeling of long range propiospinal or supraspinal neurons.

A peculiar feature found in turtles is that even in animals that recovered the hindlimbs movements, a newly formed CC was not present in the bridge region as described in regenerated spinal cords of other vertebrate species (Egar et al, 1970; Stensaas, 1983, Dervan and Roberts 2003). However, the CC of the severed cords always showed reactive signs evidenced by uneven dilatations, mainly found in segments cephalic to the injured site (not shown).

Finally, even though only 45% of the spinalized turtles recovered stepping patterns within the studied time window (up to 120 days) all showed regenerating axons crossing the injured site.

DISCUSSION

Spinal cord injury in mammals results in gliosis and limited cellular regeneration. In contrast, reliable evidence indicates that in larval cyclostomes, some teleosts and tailed amphibians there is a substantial endogenous repair that restores motor functions after spinal damage. It has been generally accepted that urodeles are the most evolved vertebrates maintaining regenerative potentialities at spinal cord levels (Stensaas 1989). Despite popular and scientific observations concerned with tail and leg regeneration in reptiles (Egar et al. 1970; Guibé, 1970) species belonging to this taxon remained unnoticed by investigators looking for more evolved animal models to study spinal cord repair after traumatic injury.

In the present article we show evidence that freshwater turtles always reconnect the transected spinal cords and given enough time about half recovered coordinated terrestrial locomotion abolished by the spinal injury. We will focus the discussion on three main issues: a) the partial functional recovery after the spinal section, b) the formation of a cellular bridge reconnecting the spinal cord stumps, and c) the invasion of the bridge by regenerating axons. Attention is mainly directed to comparative aspects, emphasizing differences and similarities between our results and that reported for other vertebrates, particularly mammals.

Functional recovery

Two or three months after complete spinal transection 45% of operated turtles recovered some of the lost motor functions, indicating that these reptiles are useful models to study endogenous repair mechanisms. However, functional recovery was always incomplete, restricted to stepping movements slower and less coordinated than those performed by normal animals. Therefore, our results indicate that even though neural reconnection occurs, not all nerve tracts and synaptic connections were fully restituted during the time window explored in this study. The fact that hindlimbs motion ceased when the animal was suspended in the air or during swimming, suggests that sensory feedback is critical for inducing stepping locomotion during recovery. Therefore, it is highly probable that the achieved motor performance may be mainly due to regeneration of sensory fibers arising from nearby ganglia outside the vertebral channel and from short propriospinal axons (see below). The failure to recover stepping locomotion in some animals despite axonal regrowth may be due to a lower general vitality after the lesion or to inadequate plastic changes in spinal excitability below the lesion.

Bridging the gap

The first physical link between the recently formed rostral and caudal stumps is the development of a clot. The presence of blood cells in direct contact with the nervous tissue is a common event that follows traumatic injury. It has been reported that in mammals, neutrophils and macrophages are the source of cytotoxic molecules triggering mechanisms for neuronal and glial death. These secondary lesions enlarge the lesion site inducing delayed demyelination (Jones et al. 2005). Our studies revealed that the clot contains, together with a predominant erythrocyte population, diverse types of leukocytes which could secrete cytotoxic agents contributing to expand the initial mechanical damage. Nevertheless, the first evidence indicating the organization of a compact cellular substrate (presence of desmosomes linking the membranes of adjacent cells) was concurrent with the presence of macrophages that phagocyte erythrocytes of the remnant clot. Therefore, in turtles cytotoxic effects mediated by macrophages do not appear as a significant obstacle to initiate cell adherence and the parallel construction of a cellular scaffold.

Since the classical studies by Ramón y Cajal (1913-14) it is accepted that reactive gliosis is one of the obvious impediments for axons to cross the lesion site. However, more recent studies have revealed that more important than the development of a mechanical glial barrier, regeneration failure is related to the presence in the lesion site of several inhibitory molecules that modulate axon growth in both normal and pathological conditions (Horner and Gage, 2000). Moreover, it is now believed that glial scarring could play a protective role precluding the invasion of still healthy tissue by potentially harmful molecules (Faulkner et al. 2004). Surprisingly, the reactive gliosis occurring in the severed spinal cord of turtles does not block the re-growth of amputated axons but seemed to contribute to direct the axons toward their targets. The BLBP+ and GFAP+ glial processes follow ordered longitudinal pathways that do not interfere with the transit of the regenerating axons. In this context, the presence in the bridge of cells expressing BLBP deserves particular attention. We have previously shown (Russo et al. 2008) that in the turtle spinal cord, a defined population of cells with the overall characteristics of RG reacts positively to the BLBP antibody. These cells are electrically coupled via connexin 43 forming functionally related clusters restricted to the lateral aspects of the CC region, where there is the highest rate of cell proliferation. BLBP+ clustered cells also expressed the transcription factor Pax 6 and thus represent a domain of neurogenic precursors in the turtle spinal cord (Russo et al., 2008). Consequently, we propose that the population of lateral BLBP+ cells found in the normal cord are a potential source for the BLBP+ cells bridging the injured site and supporting axonal growth. The early presence of BLBP+ cells within the bridge supports this idea. Furthermore, the morphological evidences suggesting some BLBP+ cells were migrating, raises the possibility that they come from the neurogenic domains around the CC in the stumps (Russo et al., 2008). In a recently published paper, Anthony et al. (2007) reported that the BLBP protein is a direct target of Notch signaling in RG during brain and cerebellum development. It seems reasonable to speculate that potential formation of new neural circuits, implying axon guidance to proper targets, should require the interplay of BLBP proteins and diverse kinds of signals that normally operate during embryonic development. The longitudinal axon-guiding channels of glial nature, described by Singer et al. (1979) during regeneration of the spinal cord of the newt, are not evident in the regenerating spinal cord of turtles.

The formation of a suitable cell support for regenerating axons is related with changes in cohesiveness of the cells. In addition to typical desmosomes, immunocytochemical procedures revealed the occurrence of ZO-1 transmembrane proteins contributing to develop intercellular adhesion. The presence of both desmosomes and tight junctions that contribute to increase cell adhesion seems paradoxical in a highly dynamic system in which cells displacements should be common. However, functions of ZO-1 protein are not reduced to stick cells or to seal extracellular pathways. Indeed, genetic evidence supports the view that ZO-1 and other proteins of the same family (MAGUKs) have a role in organizing signal transduction and help to build transmembrane protein complexes (Mitic and Anderson, 1998). Such an ample functional spectrum of ZO-1 is confirmed by a recent study (Flores et al. 2008b) indicating that ZO-1 extensively co-localizes with connexin 35 in “mixed” (electrical and chemical) synapses on Mauthner cells.

Within the poorly defined limits of the bridge and the severed stumps we have consistently observed enlarged terminals of amputated axons. They were first described by Ramón y Cajal (1913-14) in both the damaged peripheral nerves and axon tracts in the CNS of different mammalian species. Ramón y Cajal used different terms to describe them (“terminal masses”, “retraction masses”, “end or retraction balls”) suggesting that these enlarged terminals were the expression of irreversible damage. However, more recent investigations revealed that some of these dystrophic end bulbs retain sprouting capabilities either in normal (Li and Raisman, 1995) or experimental conditions (Houle and Yin, 2001).

The persistence of these unusual structures may indicate that “cytoskeletal and/or membrane plasticity must be occurring to maintain axon viability” (Silver and Miller, 2004).

Invasion of the bridge by regrowing axons

The first topic to discuss is that axonal regrowth is not mediated by the multilamellar flattened compartments usually described in embryonic or cultured nervous tissues. As described in lampreys (Shifman et al. 2007), the regenerating axon tip appeared as a cylindrical-conical compartment that lacks typical lamellipodial veils and filopodia. These peculiar axonal compartments were well stained in the spinal cord of injured turtles either with NF-M antibody or by membrane diffusion of DiI molecules.

Together with clear evidences of early axonal regrowth from dorsal roots we consistently found 5-HT+ endbulbs near the epicenter of the lesion that appeared as free endings without a recognizable post-synaptic counter part. It is now generally accepted that neurotransmitters (including serotonin) modulate axon growth and guidance to the adequate targets (Spencer et al. 1998; Koert et al. 2001; Nguyen et al. 2001). Our findings are in agreement with the proposed modulating role of 5-HT, since the release of these molecules can be viewed as an expected event, preceding and paralleling axonal invasion of the developing cellular bridge. A similar situation occurs during the development of the mammalian cortical pathways in which early serotonergic innervation is required for normal brain maturation (Mazer et al. 1997). Spinal cord transection in mammals results in the complete disappearance of 5-HT terminals in segments caudal to the lesion (Björklund and Skagerberg, 1982). In these animals, a substantial degree of locomotor recovery can be achieved by grafting embryonic serotonergic cells in the injured cord (Giménez y Ribotta, 2000). It is possible, that the serotonergic system activated in the spinal cord of the turtle could be a key endogenous mechanism that has been lost in mammals.

The existence of axons crossing the bridge was confirmed by our TEM images. At relatively early repairing stages (15 days after the lesion), we found unmyelinated axon bundles enveloped by glial lamellae. Transganglionic DiI labeling of regrowing sensory axons cast little doubts about their cells of origin and potentiality to form synaptic connections. The situation is less clear respect to the origin of other axons that were seen crossing the bridge region. That neighboring neurons lying in the rostral stump were able to cross the bridge and direct their axons toward the caudal side was revealed by retrograde labeling mediated by biocytin injections. Experiments involving extended incubation times will be needed to explore the potential regeneration of long supraspinal tracts.

Concluding remarks

There is no doubt that the spinal cord of turtles is endowed with endogenous repairing mechanisms unusual for an amniote vertebrate. This poses the question concerned with the cellular and molecular basis of these regenerative capabilities. One aspect to remark is that the clusters of functionally coupled BLBP+ cells with proliferation capabilities, and the morphology of RG (Russo et al. 2008) appear as the most plausible source for the BLBP+ cells found in the bridge region linking the cephalic and caudal stumps. Therefore, we hypothesize that the absence of a glial scar in turtles is due to the role played by the reactive BLBP+ cells that instead of forming a barrier, recapitulate an embryonic-like condition providing a permissive-growth scaffold for the incoming axons. New ideas are challenging classical concepts about commands of local spinal cord circuits by supra-spinal centers. Functions normally ascribed to “higher” nervous centers are now considered to be subserved by local spinal circuits (Bizzi et al. 2000). Taken into account these features, it is reasonable to conceive that functional recovery of spinalized turtles may be explained by the reactivation of silenced segmental circuits triggered by the arrival of massive regenerating

sensory axons. Perhaps, a serotonin rich environment promotes or regulates axon growth and the establishment of new synaptic connections. Recovery of coordinated effective stepping patterns could be explained by the neural reconnection of rostral and caudal motor generation centers without the necessary involvement of higher commands.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic drawing showing the arrangement of the anesthetic devices and the basic maneuvers to perform the spinal cord transection (not at scale). A: Turtles sedated with ketamine were deeply anesthetized with Forane®. The anesthetic was dropped into a vaporizer (V) while the head of the turtle was maintained inside a flow chamber and its legs secured to a rigid platform. Isoflurane was pressure-directed to the nostrils with the aid of a small pump (P). The spinal cord was exposed and cut at the level of the third dorsal scute. B: To test that the spinal cord was completely transected we made longitudinal sections of some of the fixed cords passing through the axial medial plane including the vertebral bodies. Note that the section of the cord was complete (arrow) from its dorsal surface to the ventral region contacting the vertebral bodies (VB). Calibration bar= 0.8 mm.

Figure 2.

Spinal cord transection induced a bilateral sensory-motor reflex limited to the hindlimbs. A: Uninjured turtles responded to tail pinching with a variety of non-stereotyped evasive movements that can involve head retraction or biting attempts. The five frames (1-5) illustrate the leg movements and the head retraction. The arrowheads indicate both the leg position and point the direction of movements. The last panel shows a schematic drawing summarizing the movements sequence in a single image. The arrow indicates the head retraction. B: Spinalized turtles exhibited the “tail-pinching reflex” that consisted of the rapid bilateral adduction of the hindlimbs ($0,49 \pm 0,05$ s) followed by their slower abduction and extension (panels 1 to 5). The arrowheads indicate both the leg position and point the direction of movements. The last panel (6) shows a schematic drawing summarizing in a single image the reflex. Double pointed arrows indicate the characteristic abduction and extension sequence.

Figure 3.

Recovery of stepping after spinal cord transection. A: Sequence of video frames (1-4) illustrating the motor behavior of a spinalized turtle 48h after spinal injury. Body motion on a solid surface was sustained exclusively by traction forces generated by the forelimbs (arrowheads). The hindlimbs remained motionless, partially retracted under the carapace (encircled). B: Sequence of video frames (1-7) recorded from another turtle that shows some recovery of hindlimbs movements 60 days after injury (the arrowheads indicate the notorious changes of position of the left leg). Panel 8 summarizes the movements of the hindlimbs as a sequence of schematic drawings of the hindlimbs (1-7). Motion direction is indicated by the lateral arrow and the superimposed outlines of the whole body. C: Video frames illustrating a spinalized turtle that recovered some coordination between forelimbs and hindlimbs 90 days after injury. Arrows in 1 to 5 indicate the motion of the right hindlimb while arrows in 6 to 10 indicate the motion of the left hindlimb. Position of the head indicates the motion of the body since the video camera was maintained motionless during recording (see complementary material).

Figure 4.

A compact blood-clot is the first link between the spinal stumps. A: Macro photograph showing the clot 28 h after spinal cord transection (asterisk). Calibration bar= 350 μm . B: Semithin section of the same material after epoxyresin embedding and section staining with boraxic methylene blue. Note the presence of early formed blood vessels (BV) containing leukocytes. Large macrophages (arrow) were intermingled with clumped erythrocytes. Calibration bar= 10 μm . C: Transmission electron microscope (TEM) image of a granulocyte included in the clot. Calibration bar= 2 μm . D: TEM images also revealed the presence of small cells with irregularly shaped nuclei and scarce cytoplasm that were tentatively identified as lymphocytes. The inset shows at a higher magnification a bundle of thin fibrils that forms part of the extracellular matrix. Calibration bars: main panel= 1 μm ; inset= 0.1 μm . E: The use of NF-M antibody revealed the presence of dystrophic endbulbs in both the cephalic and caudal stumps. Confocal optical section. Calibration bar= 30 μm . F: Enlarged view of the area encircled in E to show the uneven distribution of the NF-M+ material. Confocal optical section; calibration bar= 15 μm .

Figure 5.

A cellular bridge replaces the clot. A: Low power electron micrograph showing a large macrophage containing a phagocytosed and partially digested erythrocyte (arrow). Calibration bar= 2 μ m. B: Typical desmosomes (arrows and lower inset) were found linking adjacent cell processes. ZO-1 proteins usually associated with tight junctions completed the spectrum of cell adhesion structures (confocal microscopy; arrows in upper inset). Calibration bars: main panel= 1 μ m; lower inset= 0.25 μ m; upper inset= 10 μ m. C: Some of the numerous cell processes that form part of the first cellular scaffold were characterized by the presence of diversely oriented bundles of fine fibrils. Calibration bar= 0.5 μ m. D: Immunocytochemistry showed that cells and processes replacing the clot expressed GFAP or BLBP. Calibration bar= 20 μ m. E: Many of the BLBP positive cells bear a single thick process terminating in a conspicuous enlargement. Confocal microscopy, calibration bar= 20 μ m. F: Cells with a similar morphology were also identified with TEM. Calibration bar= 2 μ m.

Figure 6. Co-expression of BLBP and GFAP at later recovery stages. A,B,C: Double labeling experiments showing the higher expression of GFAP in the perinuclear cytoplasm and cell processes (arrows) Calibration bar = 20 μ m.

Figure 7.

Sensory axons and 5HT+ nerve terminals invade the bridge. A: Growth cones at the sites of entrance of the sensory axons (arrows). Double labeling with NF-M and GFAP antibodies revealed that these regrowing axons from dorsal root ganglion cells were accompanied by GFAP+ processes (arrowhead). The thin arrow indicates rostral direction (R). Confocal optical section, calibration bar= 25 μ m. B: Scarce 5HT+ nerve terminals (arrows) occurred near the epicenter of the lesion site. Nuclei were stained with Syto. Confocal microscopy, calibration bar= 10 μ m. C: The electron microscopy images show nerve terminals that contained vesicles with electron dense cores (arrow). Interestingly, these terminals appeared as free endings without a noticeable post-synaptic counter part. Calibration bar= 0.5 μ m.

Figure 8.

Regrowing axons populated the cellular bridge. A: Fifteen to 20 days after surgery the injured site was barely visible with the naked eye or even when examined at low magnification. The two arrows point to the cellular bridge that replaced the primitive clot. The thin arrow indicates rostral direction (R). Calibration bar= 0.8 mm. B: The bridge contained numerous unmyelinated axons partially covered by processes identified as glial sheaths (shaded blue). Electron micrograph; calibration bar= 0.25 μ m. C: Confocal microscope image showing regrowing axons stained with NF-M antibody. The arrow indicates a typical growth cone lacking filopodia and lamellipodia. The included inset (x-y orthogonal views) shows that the growth cone is not a flattened structure. Stack of 10 confocal optical sections. Calibration bar= 10 μ m. D: Two weeks after injury serotonergic varicose axons were particularly abundant at the lesion site. Stack of 15 confocal optical sections. Calibration bar= 10 μ m.

Figure 9.

Early invasion of sensory axons into the bridge. A: DiI crystals applied on cut sensory nerves close to the injured site (scheme in inset) stained few thin transganglionic DiI labeled axons travelling through the damaged region 15 days after injury. The thin arrow in the main panel indicates rostral direction (R). Confocal microscopy, calibration bar= 100 µm. B1: Cross sections passing through spinal segments caudal to the lesion also show DiI labeled axons (arrow points). To confirm the axonal nature of the axons shown in the precedent panel we combined DiI staining with NF-M antibody reaction (B2). Note the precise spatial correspondence of the DiI labeled and NF-M+ axons (arrows). Confocal microscopy, calibration bar= 20 µm. C: The lesion site was also populated by unidentified NF-M+ axons (main panel) many of which exhibited typical growing cones (inset, encircled). The thin arrow in the main panel indicates rostral direction (R). Confocal microscopy, calibration bars: main panel= 100 µm, inset =20 µm.

Figure 10.

Axons regenerated in close relation to a cellular substrate consisting of GFAP+ and BLBP+ cells. A: Longitudinal section passing through a well developed bridge (encircled) containing a high number of unidentified NF-M+ axons. The thin arrow indicates rostral direction (R). Confocal microscopy, calibration bar= 400 μ m. B: The bridge region at a higher magnification. The sections were reacted with both NF-M and GFAP antibodies. The thin arrow indicates rostral direction (R). Confocal microscopy, calibration bar= 20 μ m. C: Confocal microscope image showing that the GFAP+ processes follow longitudinal pathways paralleling the course of the NF-M+ axons crossing the lesion site. Confocal microscopy, calibration bar=10 μ m. The inset shows a transverse section of the same region. D: Double labeling with NF-M antibody and Syto nuclear stain indicates that the NF-M+ axons travel on a compact growth-permissive cellular scaffold. Confocal microscopy, calibration bar= 20 μ m. E: This panel shows one of the typical elongated BLBP+ cells found in the bridge. The section was counter stained with Syto nuclear stain. Confocal microscopy, calibration bar= 20 μ m. F-G: Near the lesion site we found some cells with a disorganized, pale cytoskeleton and others with a well stained cytoskeleton. The former were considered damaged or dead neurons while the later were considered healthy neurons that might have

survived the initial mechanical stress and the secondary cytotoxic aggression. Confocal microscopy. F, calibration bar= 15 μm . G, calibration bar=20 μm .

Figure 11.

At later stages of regeneration sensory axons are particularly abundant in the bridge. A: Application of DiI crystals on the cut surfaces of sensory nerves close to the spinal cord allowed the selective staining of dorsal roots fibers entering the spinal cord 120 days after injury. The thin arrow indicates rostral direction (R). Epifluorescence microscopy, calibration bar= 300 μ m. B: Longitudinal sections passing through the bridge show numerous DiI transganglionic labeled axons. Note the presence of growth cones (arrow). The thin arrow indicates rostral direction (R). Confocal microscopy, calibration bar= 20 μ m. C: Combination of DiI labeling with NF-M antibody revealed close contacts between DiI labeled axon terminals and medium size neurons with a well stained cytoskeleton. Confocal microscopy, calibration bar= 20 μ m.

Figure 12.

Propiospinal neurons contribute axons to the bridge. A: Application of DiI crystals on the cut surface of the spinal cord, caudal to the injured zone (yellow), resulted in the retrograde labeling of neuron perikarya at different levels of the cephalic stump (A 1-3). The thin arrow indicates rostral direction. Fixed material incubated at 37° C for 15 days. Epifluorescence microscopy, calibration bars= 10 μm. B: Iontophoretic injection of biocytin within the bridge retrogradely labeled neuronal assemblies in the rostral stump near the lesion site. C: This panel shows the cells encircled in B at a higher magnification. Transmitted light microscopy, calibration bars= 10 μm.

Table of Primary Antibodies used

Antigen	Immunogen	Manufacturer Data	Dilution
Neurofilament M (NF-M)	Recombinant fusion protein containing the C-terminal 168 amino acids of rat neurofilaments	Chemicon International Inc. (Temecula CA), rabbit polyclonal, catalog N° AB1987, Lot N° 18030413,	1:500
Glial Fibrillary Acidic Protein (GFAP)	Purified bovine GFAP	Chemicon Int. (Temecula, Ca), rabbit polyclonal, catalog N° AB 5804, lot 22010356	1:500
Glial Fibrillary Acidic Protein (GFAP)	Purified GFAP from pig spinal cord	Sigma-Aldrich, Inc. Mouse monoclonal, product N° G3893, lot: 037K4759	1:500
Serotonin (5-HT)	5-HT coupled to bovine serum albumin with paraformaldehyde	DiaSorin, Stillwater, Mi, rabbit polyclonal, catalog N° 20080, lot 051007	1:500
Brain lipid binding protein (BLBP)	Recombinant whole BLBP.	Chemicon Int. (Temecula, Ca), rabbit, catalog N° AB 9558, lot N° 6080037568	1:1000
Zonula Occludens-1 (ZO-1)	Human recombinant ZO-1 fusion protein encompassing amino acids 334-634.	Invitrogen, (Eugene OR), mouse, monoclonal Catalog No. 33-9100, lot, 50393661	1:500